Supporting Information

Experimental Procedures

1. Materials and Methods

Reagents, buffers and salts were purchased from Sigma-Aldrich, Fluka, TCI, and Acros and were used without further purification unless otherwise specified. NTPs were purchased from Fisher. The oligonucleotides were purchased from IDT and further purified by gel electrophoresis and subjected to standard desalting protocols.

T7 polymerase (P266L) was provided generously by Prof. Venkat Gopalan's lab.[1]

2. Synthetic Procedures

NTPs were purchased from Fisher. thG and ^{tz}G were synthesized according to previously published procedures.[2-3]

2.1 Hairpin RNA Synthesis

The ^{tz}G modified substrate **R1a**, thG modified substrate **R1b** were synthesized through transcription, phosphorylation and ligation.

Transcripts **1a**, **1b**, substrate **R1** as well as acceptor strand (5'-pppGGG UGC UCA GUA CGA-OH-3') were transcribed using T7 RNA polymerase (Figure S1, S4). Each single DNA template was annealed to a consensus 18-mer T7 polymerase promoter in annealing buffer (10 mM Tris-HCl, 1 mM EDTA, 100 mM NaCl, pH 7.8). The 1:1 mixture (10 μM) was heated to 90 °C for 3 min and was cooled at RT for 15 min. The transcription reaction was incubated at 37 °C for 4 h with transcription buffer (40 mM Tris-HCl, 6 mM $MgCl_2$, 10 mM NaCl, 2 mM spermidine), annealed templates (500 nM), $MgCl_2$ (14 mM), dithiothreitol (10 mM DTT), RNase inhibitor (Ribolock, 1 U/μL), and T7 RNA polymerase (0.021 μg/μL) in a total volume of 100 μL. For the native substrate **R1** and acceptor strand, ATP (2 mM), GTP (2 mM), CTP (2 mM), UTP (2 mM), and 20% v/v DMSO were used. And for the modified transcripts **1a**, **1b**, ATP (2 mM), GTP (2 mM), CTP (2 mM), UTP (2 mM), ^{tz}G/thG (10 mM in DMSO), 10% v/v DMSO were used.

Reactions were concentrated to half volume and then gel loading buffer (50 μL, 1 x TBE, 7 M Urea) was added. The mixture was denatured at 75 °C for 3 min and then each aliquot was loaded onto a preparative 20% Urea-Polyacrylamide gel. The bands were observed by UV-shadowing and the target band was excised and extracted in ammonium acetate solution (0.5 M) overnight. The gel was pelletized through centrifugation and the supernatant was loaded onto a Sep-Pak C18 column. The column was washed with 10 mL of water and the RNA was eluted with 2 mL of 50:50 water:ACN solution. The resulting solution was then centrifuged and evaporated. Solid was

dissolved in an aliquot of water and the concentration of the RNA solution was determined by UV absorption at 260 nm. The following extinction coefficients were used to calculate RNA strand extinction coefficients: C (ε_{260} = 7200 $L \cdot mol^{-1}cm^{-1}$), U (ε_{260} = 9900 $L \cdot mol^{-1}cm^{-1}$), G (ε_{260} = 11500 $L \cdot mol^{-1}cm^{-1}$), A (ε_{260} = 15400 $L \cdot mol^{-1}cm^{-1}$), ^{tz}G (ε_{260} = 5250 $L \cdot mol^{-1}cm^{-1}$), thG (ε_{260} = 5517 $L \cdot mol^{-1}cm^{-1}$). The mass of each strand was confirmed by MALDI. The yields of ^{tz}G initiated (transcript **1a**) and thG initiated (transcript **1b**) strands were 0.67 nmol / 100 µL (starting volume of reaction) and 1.12 nmol / 100 µL on average (starting volume of reaction), respectively.

2.2 5' Phosphorylation

Strands initiated with ^{tz}G/thG (transcripts **1a**/**1b**, 1-2 nmol, 6 µM) were phosphorylated at the 5'-end by T4 kinase. The reaction was incubated at 37 °C for 2 h containing kinase buffer (1x New England Biolabs), DTT (5 mM), ATP (2 mM), and T4 polynucleotide kinase (0.6 U/µL). The phosphorylated product was precipitated with cold 200 proof ethanol (2.5 times of reaction volume) and 150 µg/mL Glycoblue 0.4 M ammonium acetate placed in a dry ice bath for 1h. A blue pellet was obtained through centrifugation (14000 rpm, 20 min) and the supernatant removed. The pellet was then washed with 200 µL of 75% ethanol and dried in a speed vac for 5-10 min. Gel loading buffer (50 µL) was added to dissolve all solids before loading onto a 20% preparative Urea-polyacrylamide gel. The bands were observed by UV- shadowing and the target band was excised and extracted in 0.5 M ammonium acetate solution overnight. The gel was pelletized through centrifugation and the supernatant loaded onto a Sep-Pak C18 column. The column was washed with 10 mL of water and the RNA eluted with 2 mL of 50:50 water:ACN solution. The resulting solution was then centrifuged and evaporated. Solid was dissolved in an aliquot of water and the concentration of RNA was determined by UV absorption at 260 nm using the same extinction coefficients listed above. The mass of each strand was confirmed by MALDI. The yields of phosphorylated ^{tz}G strand and thG strand were 52% and 56% respectively.

2.3 Ligation

The extended modified RNA strands (substrate **R1a**, **R1b**) were obtained by ligation with T4 DNA ligase. Reactions were performed with a complimentary 29 nucleotide splint (Figure S6). Phosphorylated ^{tz}G / thG strand (10 µM), correlated G_{16} acceptor (10 µM) and splint (10 µM) (Figure S6) were annealed in Tris-HCl buffer (40 mM, pH 7.8) at 90 °C for 3 min and were cooled at room temperature for 30 min. $MgCl_2$ (10 mM), DTT (10 mM), PEG 4000 (0.5 mM, 0.1 v/v of 50% PEG4000) and T4 DNA ligase (1 U/µL, Fermentas) were added and the reaction was incubated at 37 °C for 2 h. The same protocol was performed as in Section 2.2 to obtain the crude ligation product as a blue pellet. Gel loading buffer (50 µL) was added to dissolve all solids before loading onto a 20%

preparative Urea-polyacrylamide gel. The bands were observed by UV-shadowing and the target band was excised and extracted in 0.5 M ammonium acetate solution overnight. The RNA was resolved by gel electrophoresis and isolated and desalted as describe above. The mass of the ligation product was confirmed by MALDI and ESI.

2.4 5' - ^{32}P Phosphorylation

The native substrate **R1**, ^{tz}G containing substrate **R1a** and thG containing substrate **R1b** were dephosphorylated by calf intestinal alkaline phosphatase (CIP). Each RNA substrate **R1**, **R1a**, **R1b** (26 pmol, 0.43 μM) was added dephosphorylation buffer (6 μL 10X) followed by CIP (2 μL) for a total volume of 60 μL and the reaction was incubated at 37 °C for 2 h. The mixture was extracted with a phenol: chloroform: isoamyl alcohol (25:24:1) solution (3 x 60 μL). The water layer was then washed with chloroform (3 x 60 μL). All aqueous layers were combined and the RNA was precipitated by adding ammonium acetate (20 μL, 10 M), glycoblue (5 μL) and 200 proof ethanol (400 μL) and put in a dry ice bath for 1 h. A blue pellet was obtained through centrifugation (14000 rpm, 20 min) and the supernatant was removed. The blue pellet was washed with 75% ethanol (200 μL) and dried in a speed vac for 5-10 min and dissolved in water (38 μL). Then the solution was added kinase buffer (5 μL, 10X), γ-^{32}P ATP (5 μL), DTT (1 μL), and T4 polynucleotide kinase (1 μL) and the solution was heated at 37 °C for 2 h. The same ethanol precipitation conditions (ammonium acetate (10 μL, 10 M), glycoblue (2 μL) and 200 proof ethanol (200 μL)) were applied to provide a blue pellet. The dried pellet was dissolved in gel loading buffer (50 μL) and loaded onto a 20 % preparative Urea-Page gel. The target band was cut out, extracted in ammonium acetate solution (0.5 M) overnight, and desalted on a Sep-Pak C18 column. The resulting solution was then concentrated using a speed vac. All solutions were stored at -20 °C.

3. Saporin Catalyzed Depurination Reaction

3.1 Gel Electrophoresis Method

Unlabeled RNA (1 μM) and a trace amount of corresponding ^{32}P-labeled RNA (2 μL, about 40,000cpm) were dissolved in 30 mM Tris-HCl reaction buffer (pH 6.0, 25 mM NaCl, 2 mM $MgCl_2$). The mixture was heated to 75 °C for 3 mins and slowly cooled down to room temperature. The mixture was placed in crushed ice for 15 mins and then brought to room temperature for 10 min. Then the mixture was prewarmed at 37 °C for 5 min before adding saporin (1.5 μM). The reaction was processed at 37 °C. Aliquots (5 μL) of the mixture were added into aniline-acetate buffer (10 μL, pH 4.5, 1 M) at scheduled time intervals (0, 5, 10, 15, 20, 25, 30 min for native substrate **R1**, 0, 5, 10, 20, 30, 45, 60, 75, 90 min for ^{tz}G modified substrate **R1a**. 0, 5, 10, 15 20, 25 ,30, 35, 40 min for thG modified substrate **R1b**). After all aliquots were taken, the mixture with aniline-acetate buffer was incubated at 37 °C for 10

min in the dark and evaporated to dryness in a Speed Vac. The product was dissolved in gel loading buffer (10 μL) before loading onto a 20 % preparative Urea-Polyacrylamide gel. The gel was analyzed by Typhoon 5 phosphorimager and the corresponding bands were quantified on ImgaeQuant TL software

3.2 RNase T1 Digestion

Each RNA substrate **R1**, **R1a**, **R1b** (2.5 μM) was mixed with a trace amount of corresponding ^{32}P-labeled RNA (1 μL, about 20,000 cpm). Yeast RNA (2 μg/μL) was added. The sample was incubated in 25 mM CEU buffer (25 mM citric acid buffer, 1 mM EDTA, 7 M urea, pH 5.0) at 50 °C for 3 min followed by the addition of 5 U of T1 RNase. The reaction was incubated in a total volume of 10 μL at room temperature for 20 min before precipitated and washed with ethanol as describe above (section 2.2). The resulting blue pellet was washed with 70% EtOH three times and was dissolved in 10 μL of gel loading buffer before loading onto a 20% preparative Urea-polyacrylamide gel. The gel was analyzed by Typhoon 5 phosphorimager.

3.3 Alkaline Digestion

Each RNA substrate **R1**, **R1a**, **R1b** (1 μM) and a trace amount of ^{32}P-labeled RNA strand (1 μL , about 20,000 cpm) was incubated in alkaline digestion buffer (25 mM sodium carbonate, 1 mM EDTA, pH 9.2) in a total volume of 15 μL at 90 °C for 15 min. An ethanol precipitation was then performed according to the protocol in section 2.2. After evaporating to dryness in a Speed Vac, the blue pellet was dissolved in 10 μL of gel loading buffer and the sample was loaded onto a 20% 7 M urea polyacrylamide gel. The gel was analyzed by Typhoon 5 phosphorimager.

3.4 Fluorescence Method

Each modified 25 μL 1 μM RNA strand (^{tz}G modified **R1a**, thG modified **R1b**) was added in 30 mM Tris-HCl reaction buffer (pH 6.0, 25 mM NaCl, 2 mM $MgCl_2$). The mixture was heated to 75 °C for 3 min and slowly cooled down to room temperature. The mixture was then placed in crushed ice for 15 min and afterwards brought to room temperature for 10 min. The mixture was prewarmed up at 37 °C for 5 min. The reaction was then initiated by adding 11 μL 1.5 μM saporin in a total volume of 125 μL. Emission spectra (360 nm-650 nm) were recorded upon excitation at 351 nm at various time points （0, 1, 3, 5, 10, 20, 30, 45, 60, 75, 90 min for ^{tz}G modified **R1a,** 0, 1, 3, 5, 10, 15, 20, 30, 40 min for thG modified **R1b**） with the excitation slit width set to 5 nm and the emission slit width set to 5 nm.

4. RNA Characterization

4.1 S1 Digestion and HPLC Analysis

All modified RNA strands (1.5 nmol) were dephosphorylated before S1 digestion. The S1 digestion was carried out in S1 reaction buffer (1x, Promega) on 1.5 nmol of RNA strands with S1 nuclease at 37 °C for 2 h in a total volume of 50 µL. The sample was concentrated to a volume of 30 µL. The ratio of each single nucleoside was obtained through HPLC Agilent 1200 series system with reverse C18 column and water: acetonitrile solvent system. All stock HPLC solvents were prepared by adding formic acid (0.1 v/v%) into MilliQ water or HPLC grade acetonitrile and then filtered through Millipore type GNWP 0.2 µm filters. Each injection was subject to a 0.5 mL/min flow rate and a gradient of 0-12% acetonitrile over 24 min. The temperature was controlled at 25.00 ± 0.10 °C The canonical nucleosides were analyzed at 260 nm. The fluorescent nucleosides ^{tz}G and thG were analyzed at 330 nm and 321 nm, respectively.

4.2 Mass Spectroscopy Characterization

MALDI spectra of the oligonucleotides were run on an Applied Biosystem Voyager-DETM Pro. The corresponding measuring matrices are illustrated in the figure caption for each oligonucleotide. The oligonucleotides were run on a ThermoFinnigan LCQ DECA XP for ESI.

5. Supporting Figures

5.1 PAGE Urea Gels

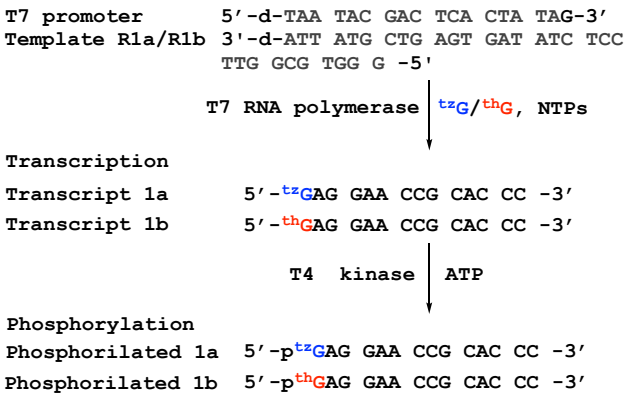


Figure S1. Pathway of synthesizing the donor of **R1a** and **R1b** strand by transcription and phosphorylation.

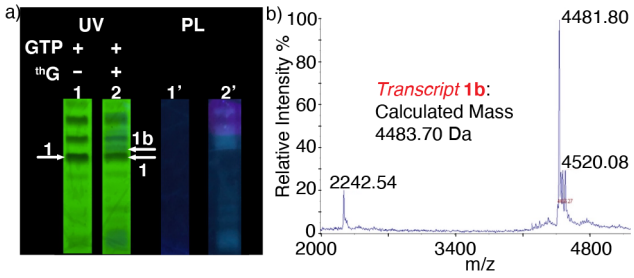


Figure S2. Transcription reactions with or without excess thG. a) Gel electrophoresis of transcription in the presence of T7 promoter, template and 2 mM NTPs with or without 10 mM thG. Lane 1 and 1': native transcription without thG. Lane 2 and 2': transcription with 10 mM thG. White arrows indicated the expected product initiated with thG and GTP. UV shadowing was observed at 254 nm and photoluminescence (PL) was observed at 365 nm. b) MALDI characterization of transcript 1b labeled by a white arrow.

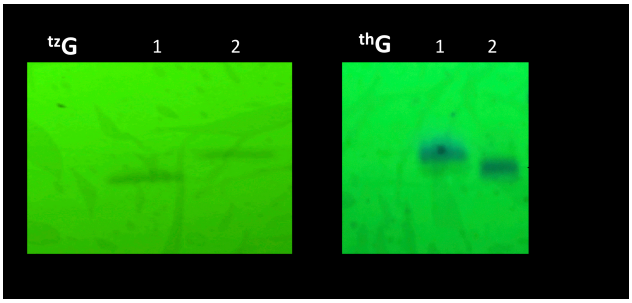


Figure S3. Phosphorylation reactions of **R1a**/**R1b** donor. For the ^{tz}G strand, Lane 1: phosphorylated product. Lane 2: starting material (transcription product initiated with ^{tz}G). For the thG strand, Lane 1: starting material (transcription product initiated with thG). Lane 2: phosphorylated product

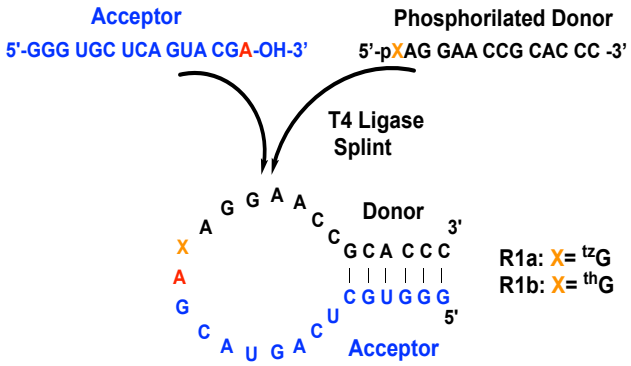


Figure S4. Ligation reactions yielding **R1a** and **R1b** strands

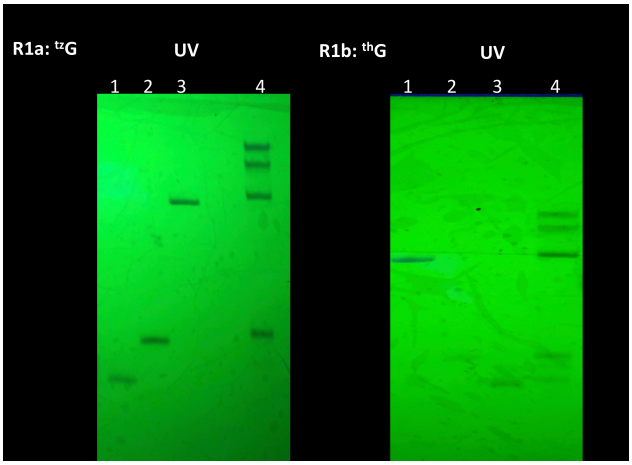


Figure S5. Ligation reactions of **R1a** and **R1b**. For **R1a**, Lane 1: phosphorylated donor of **R1a**. Lane 2: Acceptor. Lane 3: splint. Lane 4: ligation reaction. For **R1b**, Lane 1: splint. Lane 2: Acceptor. Lane 3: phosphorylated donor of **R1b**. Lane 4: ligation reaction

Ligation	
Acceptor	5′-GGG UGC UCA GUA CGA OH-3′
Donor	5′-pXAG GAA CCG CAC CC-3′
Splint	3′-CCC ACG AGT CAT GCT CTC CTT GGC GTG GG-5′
Product X= tzG or thG	5′-GGG UGC UCA GUA CGA XAG GAA CCG CAC CC-3′

Figure S6. Design and sequence of splint, donor, and acceptor

5.2 MALDI, ESI Spectra, HPLC traces and Fluorescence Spectra

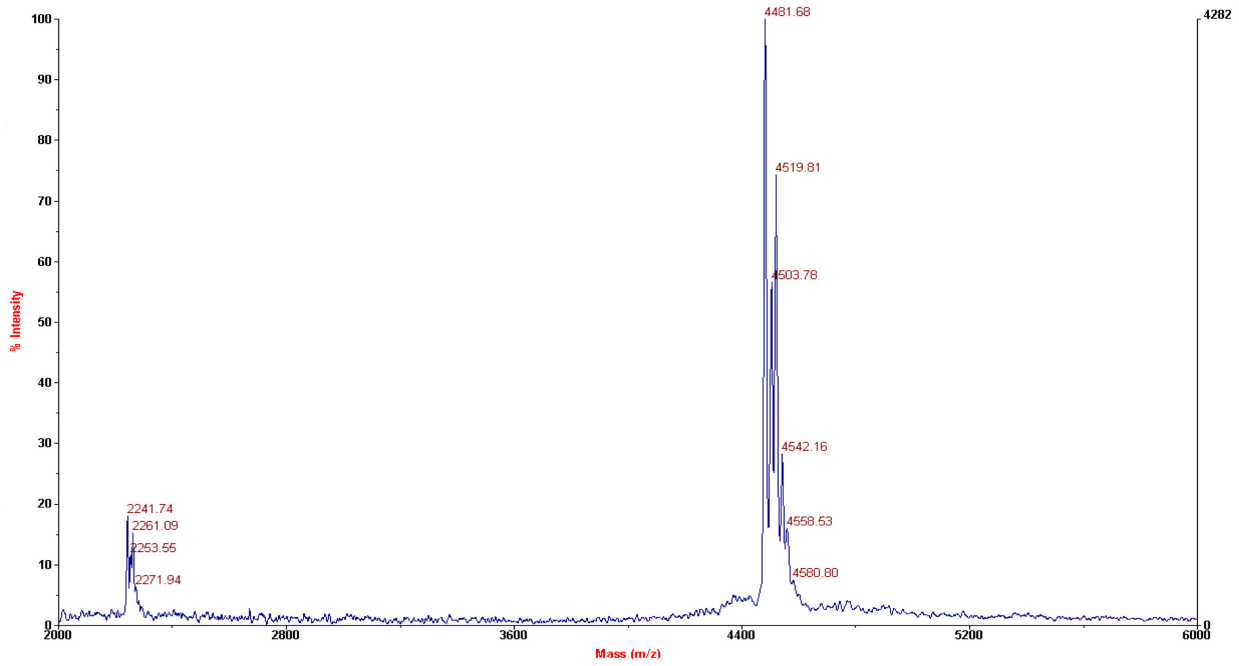


Figure S7. MALDI-TOF mass spectrum of the donor of **R1a** strand initiated with ^{tz}G. The expected molar mass was 4483.84 Da and the observed molar mass was 4481.68 Da. Characterized by MALDI-TOF mass spectrometer in negative ion mode with Voyager™ Biospectrometry™ Workstation software. All the experimental data was analyzed via Data Explorer version 4.0.0.0. THAP matrix was prepared in 1:1 water and ACN containing 20g/L 2,4,6-trihydroxyacetophenone (THAP) and 4 g/L diammonium hydrogencitrate dibasic.

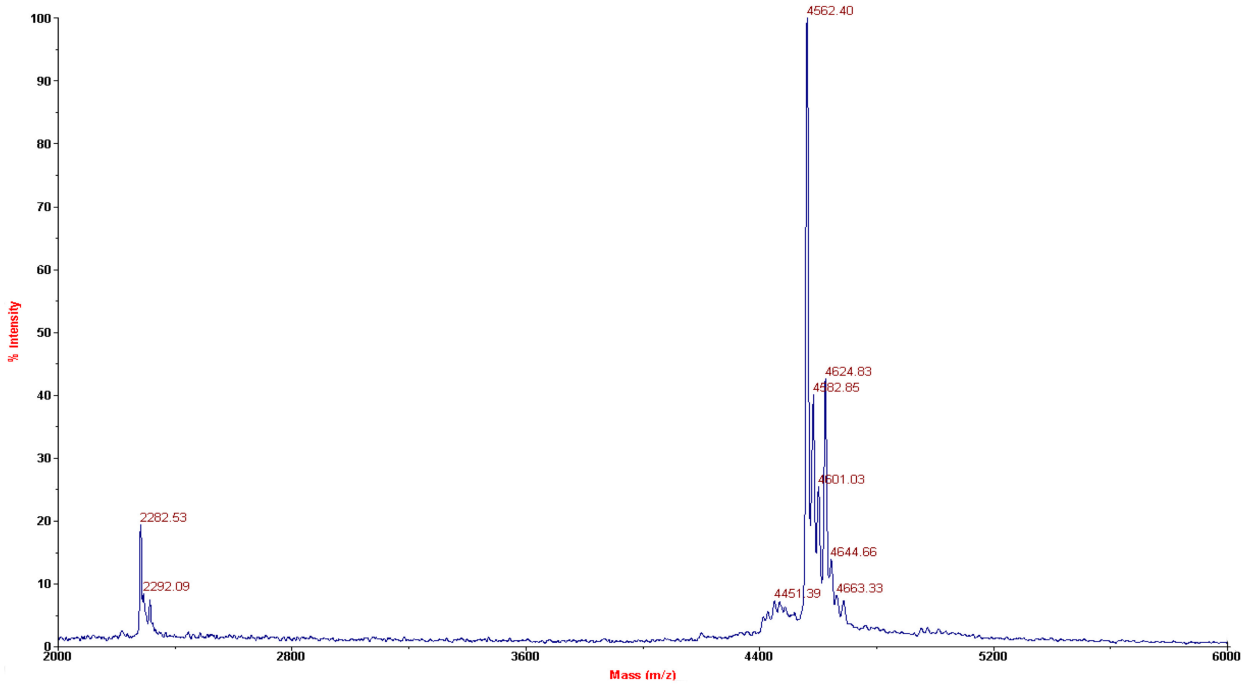


Figure S8. MALDI-TOF mass spectrum of the phosphorylated donor of **R1a** strand. The expected molar mass was 4563.84 Da and the observed molar mass was 4562.40 Da. Characterized by MALDI-TOF mass spectrometer in negative ion mode with Voyager™ Biospectrometry™ Workstation software. All the experimental data was analyzed via Data Explorer version 4.0.0.0. THAP matrix was prepared in 1:1 water and ACN containing 20g/L 2,4,6-trihydroxyacetophenone (THAP) and 4 g/L diammonium hydrogencitrate dibasic.

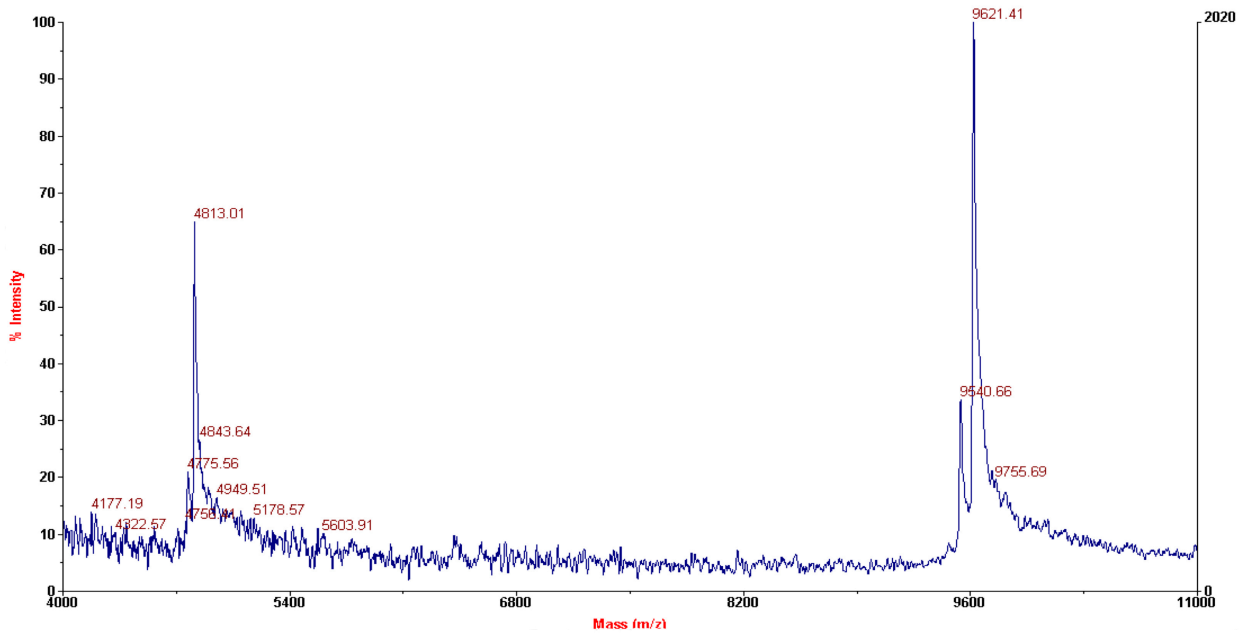


Figure S9. MALDI-TOF mass spectrum of **R1a** strand (^{tz}G strand). The expected molar mass was 9616.41 Da and the observed molar mass was 9621.41 Da. Characterized by MALDI-TOF mass spectrometer in negative ion mode with Voyager™ Biospectrometry™ Workstation software. All the experimental data was analyzed via Data Explorer version 4.0.0.0. 3-HPA matrix was prepared in 1:1 water and ACN containing 45.4 g/L 3-Hydroxypicolinic acid (3-HPA) and 10 g/L diammonium hydrogencitrate dibasic.

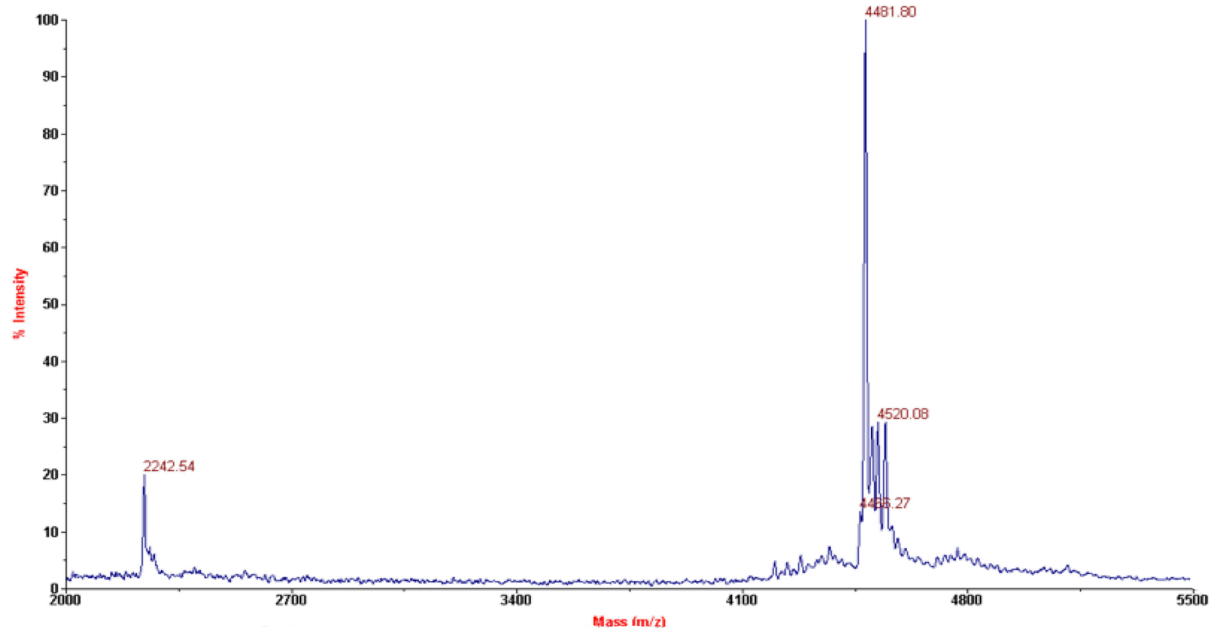


Figure S10. MALDI-TOF mass spectrum of the donor of **R1b** strand initiated with thG. The expected molar mass was 4482.84 Da and the observed molar mass was 4481.80 Da. Characterized by MALDI-TOF mass spectrometer in negative ion mode with Voyager™ Biospectrometry™ Workstation software. All the experimental data was analyzed via Data Explorer version 4.0.0.0. THAP matrix was prepared in 1:1 water and ACN containing 20g/L 2,4,6-trihydroxyacetophenone (THAP) and 4 g/L diammonium hydrogencitrate dibasic.

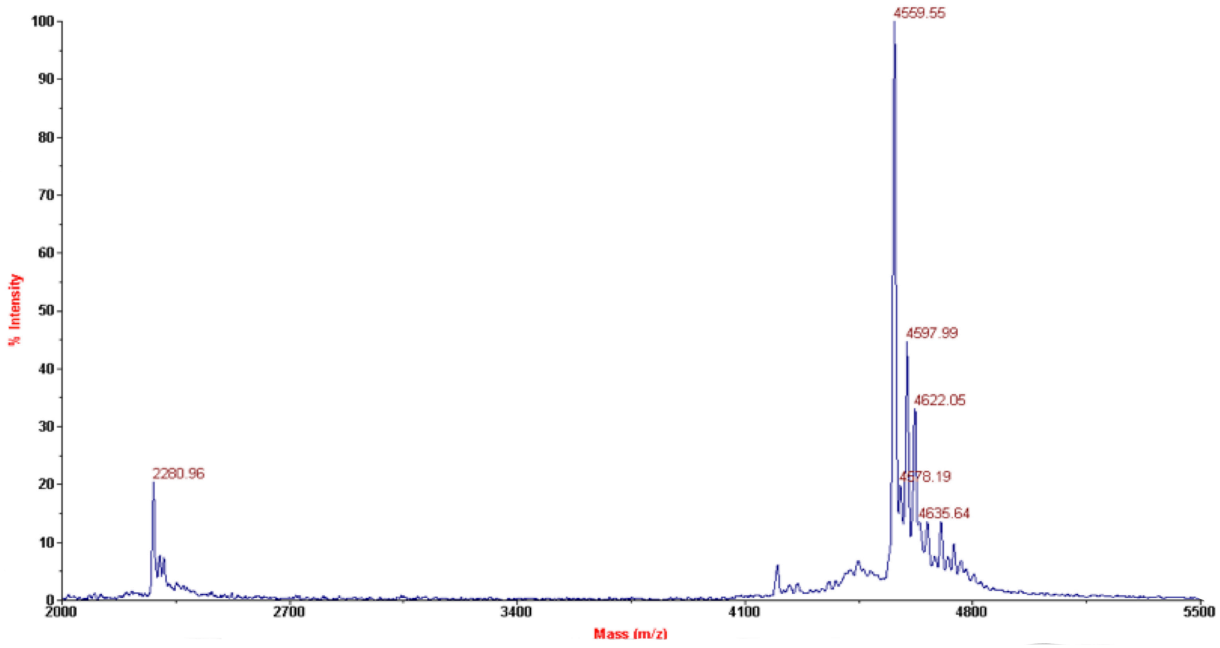


Figure S11. MALDI-TOF mass spectrum of the phosphorylated donor of **R1b** strand. The expected molar mass was 4562.84 Da and the observed molar mass was 4559.55 Da. Characterized by MALDI-TOF mass spectrometer in negative ion mode with Voyager™ Biospectrometry™ Workstation software. All the experimental data was analyzed via Data Explorer version 4.0.0.0. THAP matrix was prepared in 1:1 water and ACN containing 20g/L 2,4,6-trihydroxyacetophenone (THAP) and 4 g/L diammonium hydrogencitrate dibasic.

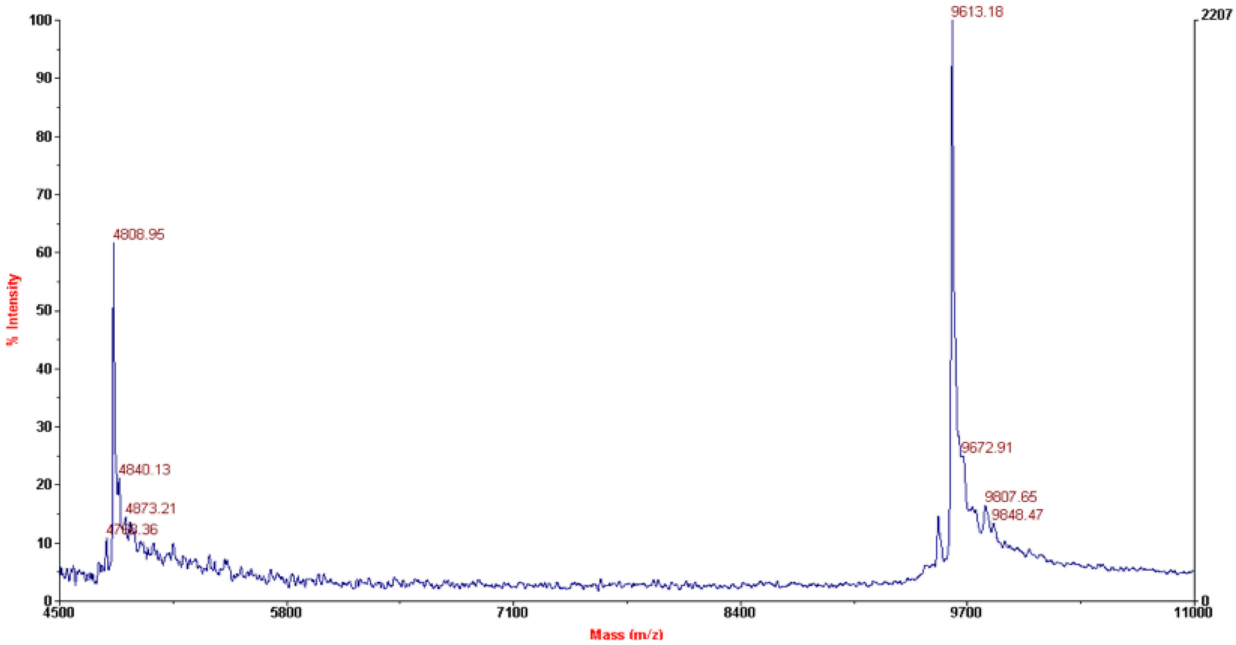


Figure S12. MALDI-TOF mass spectrum of **R1b** strand (thG strand). The expected molar mass was 9615.41 Da and the observed molar mass was 9613.18 Da. Characterized by MALDI-TOF mass spectrometer in negative ion mode with Voyager™ Biospectrometry™ Workstation software. All the experimental data was analyzed via Data Explorer version 4.0.0.0. 3-HPA matrix was prepared in 1:1 water and ACN containing 45.4 g/L 3-Hydroxypicolinic acid (3-HPA) and 10 g/L diammonium hydrogencitrate dibasic.

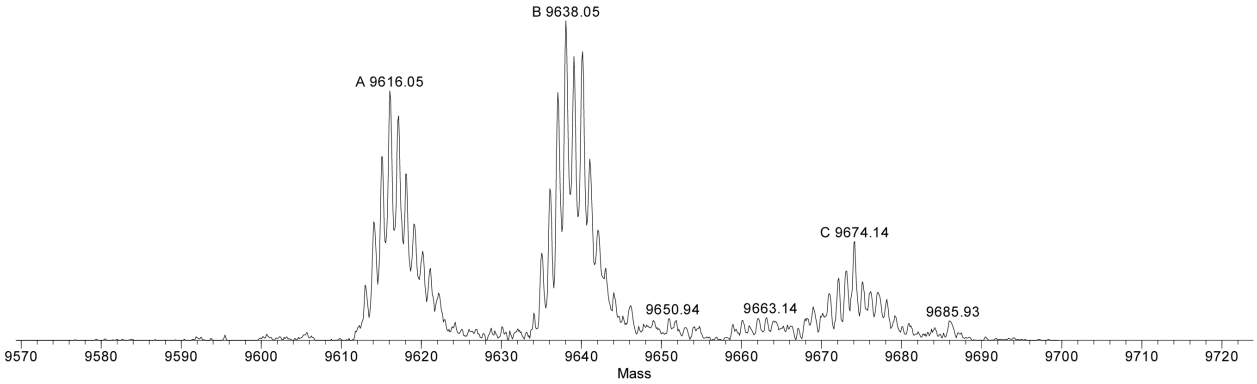


Figure S13. ESI-TOF mass spectrum of **R1a** strand (^{tz}G strand). The expected molar mass was 9616.41 Da and the observed molar mass was 9616.05 Da. Characterized by ESI-TOF mass spectrometer in negative ion mode. The raw ESI-MS m/z data were deconvoluted.

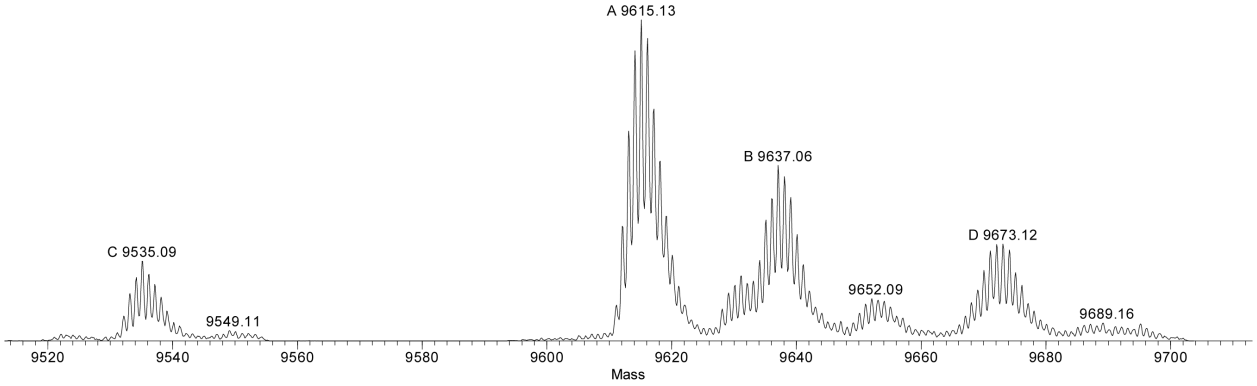


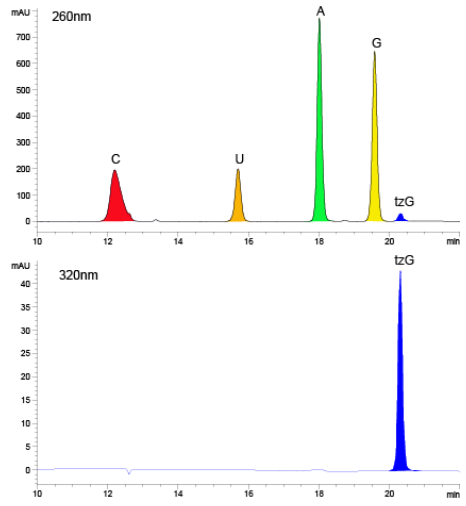
Figure S14. ESI-TOF mass spectrum of **R1b** strand (thG strand). The expected molar mass was 9615.41 Da and the observed molar mass was 9615.13 Da. Characterized by ESI-TOF mass spectrometer in negative ion mode. The raw ESI-MS m/z data were deconvoluted.

RIP substrate

R1a 5′-GGG UGC UCA GUA CGA ^{tz}GAG GAA CCG CAC CC -3′

R1b 5′-GGG UGC UCA GUA CGA thGAG GAA CCG CAC CC -3′

a)



b)

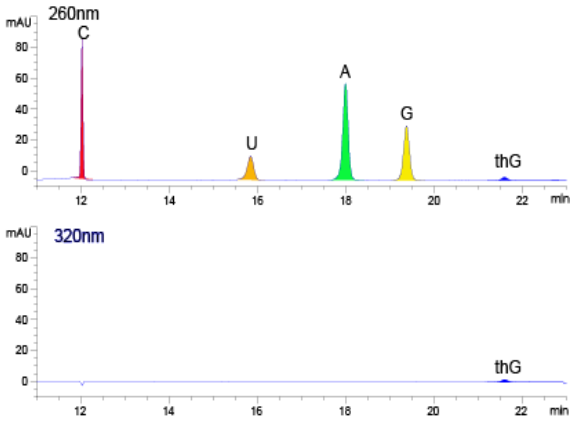


Figure S15. Characterization of strand **R1a** and **R1b**. All five nucleosides were separated by a gradient of 0–12% H_2O/Acetonitrile over 24 mins a) HPLC trace of S1 digestion of **R1a** strand and the ratio of the concentration among five nucleosides was calculated by extinction coefficient and peak area. b) HPLC trace of S1 digestion of **R1b** strand and the ratio of the concentration among five nucleosides was calculated by extinction coefficient and peak area.

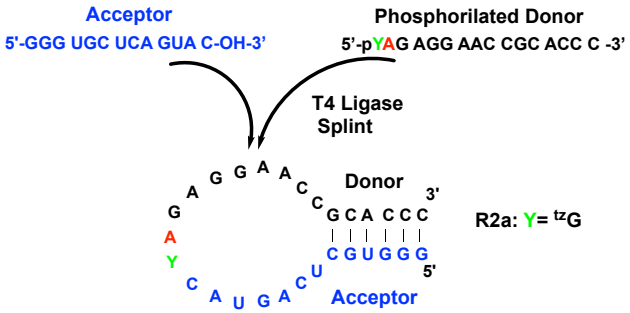


Figure S16. Ligation reactions yielding substrate **R2a**

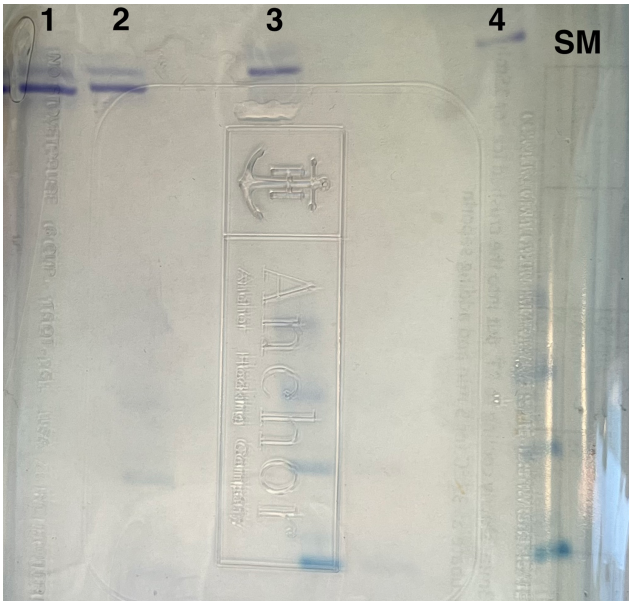


Figure S17. Stained gel after depurination of native, ^{tz}G singly substituted at G14, and treated with aniline buffer, respectively. Lane 1: native substrate **R1** without treated with aniline buffer. Lane 2: native substrate **R1** treated with aniline buffer. Lane 3: ^{tz}G modified substrate **R2a**. Reaction time: 30 min. Lane 4: ^{tz}G modified substrate **R2a**. Reaction time: 60 min.

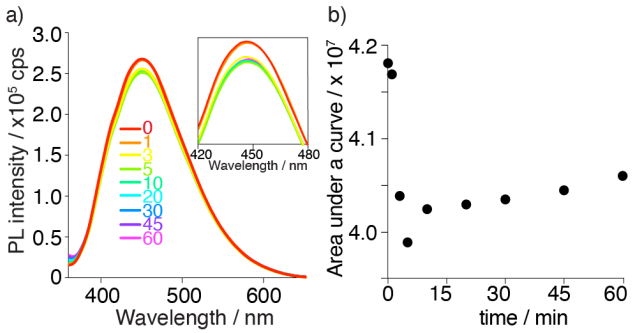


Figure S18. Saporin-mediated depurination reaction of substrate ^{tz}G **R2a** monitored by fluorescence as function of time. a) Emission spectra of depurination reaction of ^{tz}G substrate R**2a**. Excitation wavelength was 351 nm. Inset: Emission from 420 to 480 nm. b) Depurination of **R2a** by saporin at various time points monitored by fluorescence.

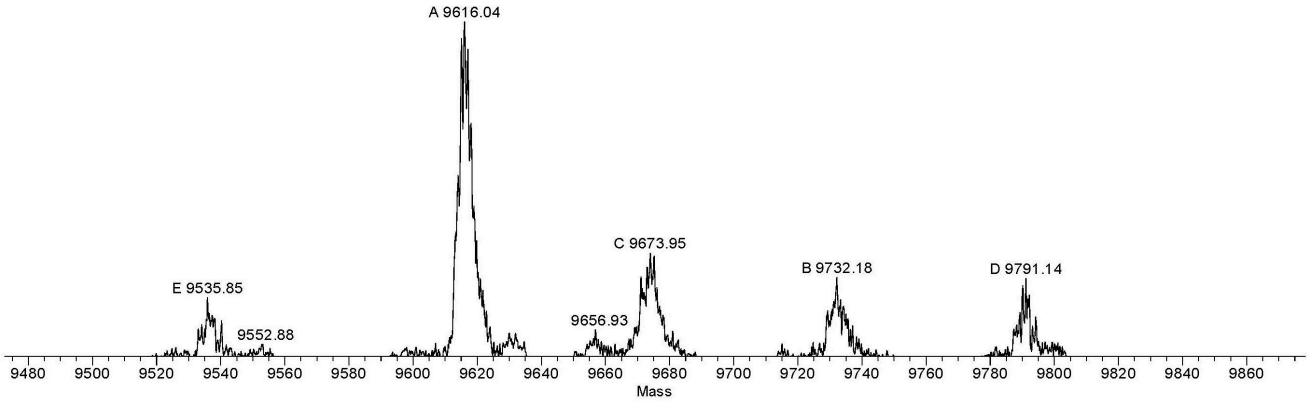


Figure S19. ESI-TOF mass spectrum of **R2a** strand (^{tz}G strand). The expected molar mass was 9616.41 Da and the observed molar mass was 9616.04 Da. Characterized by ESI-TOF mass spectrometer in negative ion mode. The raw ESI-MS m/z data were deconvoluted.

References

[1] S. Lyon, V. Gopalan, *ChemBioChem* **2018**, *19*, 142-146.
[2] A. R. Rovira, A. Fin, Y. Tor, *J. Am. Chem. Soc.* **2015**, *137*, 14602-14605.
[3] D. Shin, R. W. Sinkeldam, Y. Tor, *J. Am. Chem. Soc.* **2011**, *133*, 14912-14915.

Author Contributions

Deyuan Cong: Data Curation, Formal analysis, Investigation, Methodology, Software, Validation, Writing of Original Draft; Yao Li: Data curation, Methodology, Formal analysis, Investigation; Paul T. Ludford III: Software, Investigation; Yitzhak Tor: Conceptualization, Funding acquisition, Project Administration, Supervision, Writing for Reviewing and Editing.